

THE USE OF MEMBRANES, CELLS AND TISSUE FROM THE AREA POSTREMA
TO IDENTIFY THERAPEUTIC COMPOUNDS

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RELATED APPLICATIONS

This application claims priority from of United States Provisional Patent Application Serial No. 60/143,830, filed July 13, 1999, the contents of which are hereby incorporated in their entirety by reference.

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FIELD OF THE INVENTION

The present invention relates to the use of materials from the *area postrema* and/or adjacent sections of the brain, namely, the nucleus tractus solitarius and/or the dorsal motor nucleus of the vagus nerve. Particularly, it includes the use of membranes, cells, and/or tissue from the *area postrema* and/or these adjacent sections to identify physiologically active materials, for example materials having activity related to fuel homeostasis, by a variety of means. Such materials include proteins and chemical compounds. *Area postrema* preparations may be used to assess the ability of a material such as a protein or chemical compound to interact with naturally occurring or isolated or cloned receptor sites. More particularly, the invention relates to methods for identifying agonists and antagonists of *area postrema* biological activity, which involves assessing the ability of candidate compounds to bind to certain biologic preparations containing *area postrema* and/or adjacent brain components. Additionally, this invention relates to preparations generated from material originating from brain tissue adjacent to the *area postrema*, including materials from any part of the

nucleus tractus solitarius and/or the dorsal motor nucleus of the vagus nerve. Compounds and proteins identified in accordance with the invention may be useful in treating fuel homeostasis disorders, and may also be useful in treating 5 other metabolic and cardiorenal diseases, including diabetes, obesity, hypertension and congestive heart failure.

BACKGROUND OF THE INVENTION

The following description includes information that may 10 be useful in understanding the present invention. It is not an admission that any of the information provided herein is prior art to the presently claimed invention, nor that any of the publications specifically or implicitly referenced is prior art to or otherwise pertinent to the patentability of 15 that invention.

The *area postrema* is a small volume of tissue situated at the posterior margin of the 4th cerebral ventricle in the hindbrain. It is one of a family of circumventricular organs (CVOs) in which a fenestrated capillaries permit direct 20 communication of circulating peptides with receptors on nerve cells. The CVOs have been described as sensory organs of the brain. Receptors for peptide hormones in the *area postrema* reportedly include those for IGF-2, insulin, glutamate, 25 serotonin, substance P (NK1), arginine-vasopressin, imidazoline, angiotensin, GLP-1, NPY4, pancreatic polypeptide, PACAP, ANP, dopamine 3, melatonin, PTH/PTHrP, HCG/LH, oxytocin, VIP/secretin, somatostatin, Histamine 2, GRP, calcitonin, and amylin.

Lesioning of the *area postrema* has been reported to 30 eliminate gastric emptying effects of amylin (Edwards GL, et al., *Neurogastroenterol Motil* 1998; 10(4):365) and its satiety

effect (Lutz TA, et al., *Peptides* 1998; 19(2):309-17). Neuronal connections between vagal and other cranial nerve afferent fibers, the *area postrema*, the *nucleus of the tractus solitarius* and the dorsal motor nucleus of the vagus 5 constitute a loop that is implicated in control of gastrointestinal function. In slice preparations of the *area postrema*, the same neurons that respond to the insulin-modulating hormones, amylin and GLP-1, are reportedly responsive to glucose concentration (Riediger T, et al., 10 *Pfluegers Archiv* 437(5); Suppl R142, March 1999). Amylin-responsive neurons in CVOs, furthermore, have been reported to respond to other peptides, including Angiotensin II (Rauch S, et al., *Pfleugers Archiv.* 1997; 433 (Suppl): 619) implicated in cardiorenal control. Amylin receptors and their use in 15 various methods for screening and assaying for amylin agonist and antagonist compounds are described in co-owned United States Patent No. 5, 264,372, issued November 23, 1993.

The contents of the above-identified articles, patents, and/or patent applications, and all other documents mentioned 20 or cited herein, are hereby incorporated by reference in their entirety. Applicants reserve the right to physically incorporate into this application any and all materials and information from any such articles, patents, patent applications, or other documents mentioned or cited herein.

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SUMMARY OF THE INVENTION

The present invention relates to the use of materials, including membranes, cells and tissue from the *area postrema*, the *nucleus tractus solitarius* and/or the dorsal motor nucleus 30 of the vagus nerve, to identify physiologically active materials, for example, materials having activity in fuel

homeostasis. More particularly, the invention relates to methods for identifying materials that will stimulate or inhibit activity, or bind to preparations derived from the *area postrema*, the nucleus tractus solitarius, and/or the dorsal motor nucleus of the vagus nerve. The compounds may be useful in treating metabolic and cardiorenal diseases, including diabetes, obesity, hypertension and congestive failure.

The present invention is related in part to the surprising observation that the insulin response to infused amino acids is altered in animals with lesions of the *area postrema*. It is further based in part on the discovery that this organ is a central integrator of signals that control insulin secretion. The invention is further based on the discovery that this organ has a fuel-sensing function that ultimately drives hormonal responses important in fuel homeostasis.

Thus, the present invention provides rapid, inexpensive and physiological methods for identifying, screening and characterizing potential stimulators and inhibitors of biological action in preparations derived from any of the *area postrema*, the nucleus tractus solitarius, and/or the dorsal motor nucleus of the vagus nerve, alone or together in any combination (hereafter termed *area postrema* agonists and antagonists) for therapeutic utility. The methods comprise assessing the ability of such candidate molecules to compete against tracer concentrations of certain labeled peptides, including certain labeled peptide hormones and fragments and analogs thereof, for binding to - or activation of biological processes within - preparations from the *area postrema*, the nucleus tractus solitarius and/or the dorsal motor nucleus of

the vagus nerve. In humans, the nucleus of the tractus solitarius (NTS; solitary nucleus) is a wishbone-shaped structure subdivided in pars gustatoria and pars cardiorespiratoria. The *area postrema* is situated within the

5 v. The dorsal nucleus of the vagus nerve lies also within the

V. Nieuwenhuys, R; Voogd, J; van Huijzen, C, *The human central nervous system* (3rd edition Springer-Verlag, Berlin, 1988). In the rat the nucleus of the solitary tract is similar in shape and relationship to *area postrema* and dorsal

10 nucleus of the vagus, and has been subdivided into common, central, dorsomedial, gelatinous, interstitial, intermediate, medial and ventrolateral parts. Paxinos, G; Watson, C., *The rat brain in stereotaxic coordinates* (Compact 3rd edition, Academic Press, San Diego, 1997).

15 In one aspect, the invention provides assay methods for use in identifying or screening for *area postrema* agonists or antagonists. Such assays include bringing together a test sample and an *area postrema* preparation, the test sample containing one or more test compounds, and the *area postrema* preparation containing the various components of the *area postrema*; incubating the test sample and the *area postrema* preparation under conditions that allow the binding of, or activation by, known agonists or antagonists of *area postrema* biological function to components of the *area postrema*; and,

20 identifying those test samples containing one or more test compounds which detectably bind to, or activate, the *area postrema* preparation. An *area postrema* preparation, as indicated herein, is a preparation containing materials, typically membranes, cells, and/or tissues, from any of the

25 *area postrema*, the nucleus tractus solitarius, and/or the dorsal motor nucleus of the vagus nerve, alone or together in

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any combination. Thus, for example, a preparation containing membranes, cells, and/or tissue from the *area postrema* and the *nucleus tractus solitarius* is an "area postrema preparation." *Area postrema* preparations also include, without limitation, 5 (1) preparations containing membranes, cells, and/or tissue from the *area postrema*, (2) membranes, cells, and/or tissue from the *area postrema* and the dorsal motor nucleus of the vagus nerve, (3) membranes, cells, and/or tissue from the *nucleus tractus solitarius*, (4) membranes, cells, and/or 10 tissue from the *nucleus tractus solitarius* and the dorsal motor nucleus of the vagus nerve, and so on.

In another embodiment, this method further comprises the steps of screening test samples which detectably bind to the *area postrema* preparation for *in vitro* or *in vivo* stimulation 15 or inhibition of *area postrema* mediated activity, and identifying those test samples which act as agonists or antagonists of *area postrema* biological activity. Examples of activity assays applicable to *area postrema* preparations include electrophysiology in brain slices of *area postrema* 20 and/or associated adjacent tissues, immunohistochemical detection of activation of *area postrema* and associated tissues via detection of cFos expression, detection of intracellular cGMP-formation and NOS activity, detection of activation of *area postrema* and associated adjacent tissues 25 using a microphysiometer, and activation of *area postrema* and associated adjacent tissues detected by changes in ionic fluxes.

In a preferred embodiment, test samples which detectably bind to an *area postrema* preparation are identified by 30 measuring displacement of a labeled first ligand from the *area postrema* preparation by the test sample, and comparing the

measured displacement of the first labeled ligand from the *area postrema* preparation by the test sample with the measured displacement of the labeled first ligand from the *area postrema* preparation by one or more known second ligands.

5 Test samples used in any of the above methods that contain more than one test compound and which yield positive results can then be divided and retested as many times as necessary, and as appropriate, to identify the compound or compounds in the test sample which are responsible for yielding the

10 positive result.

In still another aspect, the invention provides for assay methods useful in determining the presence or amount of an *area postrema* preparation-binding compound in a test sample to be assayed. Such assays include an assay having steps for

15 bringing together the test sample and an *area postrema* preparation; measuring the ability of the test sample to compete against a labeled ligand for binding to components of the *area postrema* preparation; and, optionally, relating the amount of *area postrema* preparation-binding compound in the

20 test sample with the amount of *area postrema* preparation-binding compound measured for a negative control sample, the negative control sample being known to be free of any *area postrema* preparation-binding compound, and/or relating the amount of *area postrema* preparation-binding compound in the

25 test sample with the amounts of *area postrema* preparation-binding compound measured for positive control samples which contain known amounts of *area postrema* preparation-binding compound, in order to determine the presence or amount of *area postrema* preparation-binding compound present in the test

30 sample. This assay method, in still further embodiments, can

be utilized to evaluate the characteristics of an *area postrema* preparation.

In another aspect, components of the *area postrema* preparations of the invention may also be bound to a solid 5 phase and used in various affinity chromatography methods and used, for example, for the purification of compounds capable of binding to components of the *area postrema* preparation or the evaluation of samples known or suspected to contain such compounds, or agonists or antagonists of such compounds.

10 As noted above, the invention may utilize *area postrema* material as well as material originating from brain tissue adjacent to the *area postrema*, i.e., the nucleus tractus solitarius and dorsal motor nucleus of the vagus nerve.

15 DETAILED DESCRIPTION OF THE INVENTION

The present invention includes methods for screening, identifying, and characterizing potential compounds that bind to, or stimulate or inhibit activity of, the *area postrema*, for example compounds having activity in fuel homeostasis, 20 which comprises assessing the relative abilities of candidate agonists and antagonists to compete against relevant chemical compounds for binding to components of *area postrema* preparations.

The present invention is based, at least in part, on the 25 surprising discovery that the *area postrema*, like the pancreatic islet, is a fuel-sensing organ, and that agents that interact with this tissue will evoke responses that are potentially useful in the treatment of disorders of fuel homeostasis. The same structures involved in fuel sensing may 30 also be involved in cardiorenal control, and agents that act

there to restore metabolic functions will also be useful in restoring normal cardiorenal function.

One such method for use in identifying or evaluating the ability of a compound to play a role in fuel sensing involves 5 bringing together a test sample and a test system, the test sample containing one or more test compounds, the test system containing a system for evaluating fuel sensing.

The following detailed description of the invention includes techniques for membrane preparation, binding 10 reactions, data detection and data analysis. It also includes techniques for evaluating the activity of *area postrema* preparation-binding compounds. Additionally, this description includes techniques for pharmaceutical formulation and delivery of test substances. Finally, a number of 15 applications of this invention are described, including its use in high throughput assays and assays to characterize agonists and antagonists of *area postrema* function.

I. Membrane Preparation

20 In this particular embodiment, *area postrema* preparations are prepared using membranes. *Area postrema* membrane preparations are initiated by brief (4-10 seconds) homogenization of tissues at ice bath temperatures at a buffered pH of about neutrality. In one embodiment, an 25 instrument such as a Polytron (Brinkman Instruments, N.Y.) is used, although other similar homogenizers may also be used. Following tissue disruption, membranes are isolated in the cold at g-forces of at least about 20,000 x g for an appropriate time, preferably above 40,000 x g for at least 10 30 minutes. Membranes are normally washed at least twice by re-homogenization in fresh buffer, and reisolated as above, in

order to remove endogenous interfering substances. Washed membranes are resuspended in buffer containing a proteolytic enzyme inhibitor such as phenylmethylsulfonyl fluoride (PMSF) or bacitracin. Volumes of buffer may be added sufficient to 5 adjust the final tissue concentration to a level suitable for the particular screening method embodiment employed.

As indicated herein, cells and/or tissue samples may also be utilized to make *area postrema* preparations. Cells and/or tissue samples are prepared by art-known methods.

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II. Binding Reactions

In one embodiment, incubation mixtures for the screening method are set up as follows. To glass or polymeric tubes are added a small volume of Buffer Mixture ("HBBM") composed of a 15 buffer solution such as HEPES containing a protease inhibitor such as bacitracin or PMSF, protease-free serum albumin (preferably fraction V BSA, protease-free) and, optionally, a Mg^{2+} salt. To this Buffer Mixture is added a small volume of buffer containing the unlabeled molecules to be tested for 20 agonist or antagonist activity at concentrations of about from 10^{-11} to 10^{-6} M. Control tubes contain buffer alone. To this mixture is added amounts of labeled *area postrema* preparation ligands, in buffer so as to produce final concentrations of from about 10 to about 100 pM. Because of the high specific 25 activities obtainable and ease of chemical labeling, ^{125}I is preferred to label the *area postrema* ligands. The peptide hormones may be isolated from human tissues, from animal tissues, or produced by chemical synthetic or recombinant means.

Labeled *area postrema* preparation ligands are dissolved in sterile water containing protease-free Fraction V BSA, aliquoted, and stored frozen until use.

Reactions are begun by adding, for example, membranes to 5 each incubation tube. The amount of tissue (or, more conveniently, the amount of membrane protein) required per tube will be dictated according to tissue type. Typically, membranes from about 2.5 mg of tissue (about 100 μ g membrane protein) are added.

10 Reaction mixtures are incubated for a period of time and at a temperature sufficient to reach steady-state conditions within the period. The term "steady state" as used herein is intended to encompass the sum total of all reactions and processes that influence the net amount of bound hormone. It 15 may or may not be synonymous with "equilibrium." Typically, tubes are incubated for about 60 minutes at room temperature.

III. Detection

When membranes are used, they are isolated following 20 binding in order to determine the amount of labeled ligand bound after competition between labeled and unlabeled ligands. It is convenient to collect membranes by filtration with a vacuum-powered Brandel Cell Harvester (Brandel Instruments, Gaithersburg, Maryland, Model M-24) through glass fiber 25 filters (e.g., GF/B, Whatman) that have been presoaked with a reagent in order to reduce nonspecific binding (NSB). Preferred is presoaking filters for about 5 hours in about 0.3% polyethyleneimine. The skilled artisan will know of other plasma membrane collecting devices, such as the 30 Millipore Filtration Assembly (Model 1225) or the Sandbeck filter box (Bennett, J.P., in Neurotransmitter Receptor

Binding, H.I. Yamamura *et al.*, Raven, New York 1978, pages 57-90), collecting filters, and NSB-reducing reagents that can be used in practicing this invention. Both immediately before and immediately after filtration, filters are washed with

5 large (milliliter) volumes of ice cold buffer to remove contaminating materials, *e.g.*, unbound labeled ligand.

Filters are removed and the amount of labeled ligand bound to plasma membranes is quantified. Where ^{125}I is the label,

radioactivity may be assessed in a gamma ray counter. Where a

10 chemiluminescent reporter molecule (*e.g.*, AMPPD, Tropix, Inc., Bedford, MA) is used, the light produced may be quantified in a luminometer. Enzymatic and fluorescent labels may also be used.

Instead of by filtration, plasma membranes may be

15 isolated following incubation by centrifugation (*e.g.*, Beckman J-2-21-M refrigerated centrifuge at 21,000 rpm or a Beckman 12 or Eppendorf microfuge), washed with ice cold buffer, then counted as such or following solubilization of membranes by detergent or alkali.

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IV. Data Analysis

Scatchard plot saturation analyses of binding data, wherein bound/free (B/F) labeled ligand is plotted as a function of the amount bound, are performed by standard

25 methods. See, *e.g.*, Blecher 1976, Blecher 1981, Chapter 1, and Boulton *et al.* 1986, Chapter 1.

Competition curves, wherein the amount bound (B) is plotted as a function of the log of the concentration of ligand may be analyzed by computer, *e.g.*, analyses by 30 nonlinear regression to a 4-parameter logistic equation (Prism program; GraphPAD Software, San Diego, California) or the

ALLFIT program of DeLean et al. (ALLFIT, Version 2.7 (NIH, Bethesda, MD 20892)). Munsun, P.U. and Rodbard, D., Anal. Biochem. 107:220-239 (1980).

To determine binding constants, Scatchard saturation curves may be generated and analyzed according to a modification of the method of Scatchard, as described by Bylund, D.B., et al., "Methods for Receptor Binding," in H.I. Yamamura et al., eds., Methods in Neurotransmitter Analysis, Raven Press, New York, 1990 pp. 1-35.

In order to obtain specific binding values experimentally, a broad range of tracer concentrations of labeled ligand (typically, 1-150 pM) is used to obtain total binding and duplicate tubes reassessed, in the presence of a very high concentration, e.g., 100 nM, of unlabeled ligand, to obtain nonspecific binding (NSB). The latter value is subtracted from each total binding value in order to obtain specific binding at every concentration of labeled ligand.

V. Activity of Area Postrema Preparation-Binding Compounds

Examples of activity assays useful for detecting the activity of compounds that bind to *area postrema* preparations include electrophysiology in brain slices of *area postrema* and/or associated adjacent tissues, immunohistochemical detection of activation of *area postrema* and associated tissues via detection of cFos expression, detection of intracellular cGMP-formation and NOS activity, detection of activation of *area postrema* and associated adjacent tissues using a microphysiometer, and activation of *area postrema* and associated adjacent tissues detected by changes in ionic fluxes.

In a method for performing electrophysiology in brain slices of *area postrema* and associated adjacent tissues, extracellular recordings are made from brain slices approximately 0.5mm thick of *area postrema*. Slices are 5 typically superfused with artificial cerebrospinal fluid (aCSF) of the following composition (in mM): NaCl 124; KCl 5; KH₂PO₄ 1.2; MgSO₄ 1.3; CaCl₂ 1.2; NaHCO₃ 26; glucose 10; pH: 7.4, equilibrated with 95% O₂ and 5% CO₂, 290 mOsm/kg, at 37.0°C. Extracellular electrodes are used to record 10 spontaneous or stimulated action potentials of neurones in the slice. Signals from electrodes are suitably amplified and processed by signal discriminators and counters to derive measures of neuronal activity. Test substances are applied via the superfusion. Additionally, stimuli may be applied 15 that identify the activity recorded from the brain slice as being relevant to metabolic control, such as responsiveness to changes in ambient glucose (eg steps of 2, 4 or 6mM) or other fuels.

In a method for performing immunohistochemical detection 20 of activation of *area postrema* and associated adjacent tissues via detection of cFos expression, neuronal activation is associated with the induction of a protein, cFos. Appearance of this protein, or of RNA coding for its expression, can be used to indicate that a structure has been activated. To test 25 if a compound activates *area postrema* and associated tissues, the test substance (or a control) is administered to a live animal. Following a suitable period to allow induction of cFos expression, for example 90 min, animals are anaesthetized and perfused transcardially with 4% paraformaldehyde (PFA). 30 Brains are removed and cryoprotected by 24h incubation in phosphate buffer containing 10% sucrose before cutting 20 µm

thick cryosections. Sections are then incubated for 48 h on poly-L-lysine covered slides in phosphate buffered saline containing an antibody directed against cFos protein.

5 Immunoreactivity was detected performing the avidin-biotin-peroxidase method. Differences in cFos immunoreactivity in *area postrema* and associated tissues between brains from animals treated with test substances versus control substances can be used to identify substances activating those structures.

10 In a method for detecting intracellular cGMP-formation and NOS activity, activation of *area postrema* and associated adjacent tissues can be detected by measuring changes in intracellular second messenger. For example, where cyclic GMP mediates activation via surface receptors, *area postrema* activation can be detected either *in vitro* or *in vivo*. For *in vivo* studies, a test substance or saline (control) is 15 subcutaneously injected into rats which have been pretreated with intraperitoneal injection of 10mg/kg 3-isobutyl-1-methylxanthine (IBMX) to inhibit degradation of the second messenger. Some time after administration of the test 20 substance (eg 25 min), rats are perfused and cryosections of the brain are cut. For *in vitro* studies, a slice preparation of *area postrema* is made as for electrophysiological recordings. Slices are incubated at 37°C in 2 ml oxygenated 25 aCSF containing 1mM IBMX. Test substances at a range of concentrations are added for between 10 and 40 min. Effects of test substances on generation of cyclic GMP are then assessed immunohistochemically. A similar test can be employed for detection of activation of other second 30 messengers, such as nitric oxide, by immunodetection of nitric

oxide synthetase (NOS) activity by NADPH-diaphorase staining at *area postrema* and associated adjacent tissues.

With regard to detecting activation of *area postrema* and associated adjacent tissues using a microphysiometer, it is understood that changes in cellular activity, such as following stimulation with a ligand, typically result in changes on rate of energy metabolism. General activation of cells in culture, dissociated cells, subcellular components, or slices of tissues can be detected by the rate at which they produce acid, a byproduct of metabolism. Changes in rates of acidification, indicative of activation, may be detected in preparations from *area postrema* and associated tissues using a physiometer.

With regard to activation of *area postrema* and associated adjacent tissues detected by changes in ionic fluxes, it is also known that signals impinging upon nerve cells typically affect processes that are associated with controlling conductances through various ion channels that traverse the cell membrane or other cellular compartments. Activation of neuronal tissue can be detected by changes in ionic fluxes, and these latter events can be detected via a variety of means, including by electrophysiologic recordings (intracellular, extracellular and by patch clamping) and by other means of detecting changes in voltage or current, such as with dye-based systems that are sensitive to changes in charge distribution (eg voltage-sensitive dyes). Other detection systems may be sensitive to the presence of particular ionic species, such as with calcium-sensitive dyes. Other systems can indirectly report ionic events via induction of reporter genes whose products can be easily detected, for example, by color change. Systems such as these are used

singly, or in combination, to detect activation of preparations from *area postrema* and associated adjacent tissues.

5 VI. Pharmaceutical Formulations / Routes of Delivery

Compounds identified using the methods of the present invention will be useful as agents to modulate *area postrema* function. Formulations and dosages for such compounds as described herein are useful in view of their pharmacological 10 properties. *Area postrema* preparation-binding compounds may conveniently be provided in the form of formulations suitable for parenteral (including intravenous, intramuscular and subcutaneous) administration. Also described herein are formulations and dosages useful in alternative delivery 15 routes, including oral, nasal, buccal, sublingual and pulmonary.

Area postrema preparation-binding compounds useful in the invention can be provided as parenteral compositions for injection or infusion. They can, for example, be suspended in 20 an inert oil, suitably a vegetable oil such as sesame, peanut, olive oil, or other acceptable carrier. Preferably, they are suspended in an aqueous carrier, for example, in an isotonic buffer solution at a pH of about 5.6 to 7.4. These compositions may be sterilized by conventional sterilization 25 techniques, or may be sterile filtered. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH buffering agents. Useful buffers include for example, sodium acetate/acetic acid buffers. A form of repository or "depot" 30 slow release preparation may be used so that therapeutically effective amounts of the preparation are delivered into the

bloodstream over many hours or days following transdermal injection or delivery.

Desired isotonicity may be accomplished using sodium chloride or other pharmaceutically acceptable agents such as 5 dextrose, boric acid, sodium tartrate, propylene glycol, polyols (such as mannitol and sorbitol), or other inorganic or organic solutes. Sodium chloride is preferred particularly for buffers containing sodium ions.

Area postrema preparation-binding compounds can also be 10 formulated as pharmaceutically acceptable salts (e.g., acid addition salts) and/or complexes thereof. Pharmaceutically acceptable salts are non-toxic salts at the concentration at which they are administered. The preparation of such salts can facilitate the pharmacological use by altering the 15 physical-chemical characteristics of the composition without preventing the composition from exerting its physiological effect. Examples of useful alterations in physical properties include lowering the melting point to facilitate transmucosal administration and increasing the solubility to facilitate the 20 administration of higher concentrations of the drug.

Pharmaceutically acceptable salts include acid addition salts such as those containing sulfate, hydrochloride, phosphate, sulfamate, acetate, citrate, lactate, tartrate, methanesulfonate, ethanesulfonate, benzenesulfonate, p- 25 toluenesulfonate, cyclohexylsulfamate and quinate.

Pharmaceutically acceptable salts can be obtained from acids such as hydrochloric acid, sulfuric acid, phosphoric acid, sulfamic acid, acetic acid, citric acid, lactic acid, tartaric acid, malonic acid, methanesulfonic acid, ethanesulfonic acid, 30 benzenesulfonic acid, p-toluenesulfonic acid, cyclohexylsulfamic acid, and quinic acid. Such salts may be

prepared by, for example, reacting the free acid or base forms of the product with one or more equivalents of the appropriate base or acid in a solvent or medium in which the salt is insoluble, or in a solvent such as water which is then removed 5 in *vacuo* or by freeze-drying or by exchanging the ions of an existing salt for another ion on a suitable ion exchange resin.

If desired, solutions of the above compositions may be thickened with a thickening agent such as methyl cellulose. 10 They may be prepared in emulsified form, either water in oil or oil in water. Any of a wide variety of pharmaceutically acceptable emulsifying agents may be employed including, for example, acacia powder, a non-ionic surfactant (such as a Tween), or an ionic surfactant (such as alkali polyether 15 alcohol sulfates or sulfonates, e.g., a Triton).

Area postrema preparation-binding compounds useful in the invention are prepared by mixing the ingredients following generally accepted procedures. For example, the selected components may be simply mixed in a blender or other standard 20 device to produce a concentrated mixture which may then be adjusted to the final concentration and viscosity by the addition of water or thickening agent and possibly a buffer to control pH or an additional solute to control tonicity.

The optimal formulation and mode of administration of *area 25 postrema* preparation-binding compounds identified in accordance with the present application to a patient depend on factors known in the art such as the particular disease or disorder, the desired effect, and the type of patient. While the compounds will typically be used to treat human patients, they 30 may also be used to treat similar or identical diseases in other vertebrates such as other primates, farm animals such as

swine, cattle and poultry, and sports animals and pets such as horses, dogs and cats.

VII. Utility

5 The results of the Examples below establish that the method described herein for measuring the ability of chemical compounds to compete against labeled known ligands for binding to specific components of an *area postrema* preparations represents a particularly useful means for identifying 10 peptides and other chemical compounds that interact with such preparations. Certain of the utilities of the invention described and claimed herein are further highlighted below.

A. Measurement of Test Compounds

15 In one aspect, the Examples further demonstrate that *area postrema* preparation assays can be used to determine the concentration of a test compound in unknown solutions or mixtures. For example, test compounds may be assayed as described in Example II below. A membrane or cell preparation 20 of *area postrema* components, for example, is incubated with radiolabelled test compound and unlabelled test compound at concentrations of 10^{-6} M. In this manner, a competition curve is generated relating the amount of test compound in the assay 25 tube to the inhibition of radiolabelled test compound binding produced. In additional tubes, unlabelled peptide is replaced by a solution containing an unknown amount of test compound to be quantified. This solution may be plasma, serum or other fluid, or solid mixture dissolved in assay buffers. The unknown solution is preferably added in a volume of less than 30 or equal to about 10% of the final assay volume, so as not to significantly alter the ionic content of the solution. If

larger volumes of unknown are used, a solution containing an equivalent salt content is included as a control for effects of altered ionic content on binding. Nonspecific binding, i.e., binding of radiolabelled test compound in the presence 5 of a high concentration (10^{-6} M) of unlabelled test compound or other known binding compound, is subtracted from total binding for each sample to yield specific binding. The amount of inhibition of specific binding of radiolabelled test compound produced by the unknown is compared to the inhibition curve 10 produced by test compound in order to determine the content of substances capable of binding *area postrema* components in the unknown sample. Methods for performing these calculations are described in several sources, such as Neurotransmitter Receptor Binding, eds H. Yamamura, S.J. Enna, and M.J. Kuhar 15 (Raven Press, New York, 1991).

This method is used to quantitate the amount of *area postrema* binding compounds in a known or an unknown sample, and may be used to quantitate *area postrema* binding compounds in plasma or other body fluids and tissues, for use in 20 identifying active metabolites, pharmacokinetics, stability, solubility, or distribution of test compounds, agonists and test compound antagonists. In order to increase the specificity of the assay for the test substance where this is necessary, the quantity of other binding substances in the 25 unknown sample can be determined through a radio-receptor assay for these substances. Such a radio-receptor assay can be performed using known ligand that has been radiolabeled, an *area postrema* membrane preparation and the unknown test sample.

B. High Throughput Screens

In still another aspect, the *area postrema* component preparations are used in a high throughput screen, optionally utilizing robotic systems such as those known in the art, for identifying compounds that activate various biological processes, or which displace known compounds, sometimes referred to by some as "ligands," from their binding sites and, thus, identifying candidate *area postrema* agonists or antagonists. The assay can be used to screen, for example, libraries of synthetic compounds, extracts of plants, extracts of marine organisms, or bacterial or fungal fermentation broths. In one embodiment, an initial step brings together about 50fL of the *area postrema* preparation described above, pre-incubated with about 10 to about 15 pM labeled known ligand as described above, and approximately 50fL of the solution of test compound, in assay buffer containing, for example, up to 10% ethanol, or 1% DMSO, or 5% acetonitrile to facilitate dissolution of compound, if required. For organic extracts, the final concentration of solvent should generally not exceed that which displaces the standard displacement curve of labeled ligand by cold compound by 25%, i.e., shifts the measured IC₅₀ by less than 25%. This can be evaluated for each selected solvent. For identified compounds from synthetic libraries, the test concentration will be about 100nM, 1μM or 10μM depending on the frequency with which positive tests occur. A positive will typically be represented by at least about a 20% reduction of specific binding of labeled ligand. With broths and extracts, a positive test will be denoted by at least about 20%, 50% or 80% reduction in specific ligand binding, according to the frequency of positive tests.

For compounds meeting defined criteria, the potency of interaction with the *area postrema* is determined by measuring the magnitude of activation of a biological process, or the displacement of ligand from the membrane or other preparations by a range of concentrations of the test compound. With mixtures of unknown compounds, as in broths and extracts, the desired activity is isolated and purified by art-known methods including HPLC, followed by testing the separated materials to determine which retain the desired activity. When pure or relatively pure active material is obtained, its potency at the *area postrema* can be determined. Art-known methods including NMR, mass spectroscopy, and elemental analysis may be used to make a chemical identification of any isolated material having the desired *area postrema* activating and/or binding activities.

At any desired stage following identification of selective displacement of ligand, a positive testing material can be assessed in a functional assay to assess *area postrema* agonist activity. Activity assays such as those described above may be used. Also, by applying different concentrations of the test material in these assays, the potency of agonist or antagonist action can be determined.

C. Agonists and Antagonists

In other embodiments, for assessment of whether materials testing positive in an *area postrema* preparation binding assay are agonist or antagonists, the test materials are brought together with, for example, *area postrema* membrane or cell systems in which known ligand changes rates of synthesis of cyclic AMP. Such preparations include membranes prepared from cultured cell lines with abundant *area postrema* components, or

the cells themselves. Changes in cAMP levels are measured by radioimmunoassay following exposure of the membrane or cell preparations, incubated according to art-known methods. In another aspect, other indicators of cellular response may be 5 used. Examples of analytes include cyclic GMP, NO, intracellular Ca^{++} . More general indicators of cellular response include change in rate of acidification, as measured in a microphysiometer, changes in membrane potential, as measured by intracellular electrodes or voltage-sensitive 10 dyes, and change in neuronal activity, as indicated by changes in firing rate detected with extracellular electrodes. Also included in cellular responses are changes in rate of gene transcription and expression as measured, for example, by the appearance of various functional or reporter products.

15 Materials testing positive in displacing known ligands from its receptors and having no effect on second messenger production can be expected to be *area postrema* antagonists. Antagonist action can be further evaluated by incubating various concentrations of the material analog with known 20 ligand or a known ligand agonist and measuring the degree of inhibition of the changes in cAMP evoked by the known ligand or an known ligand agonist.

In another aspect, the invention is used to screen 25 materials for *area postrema* components. Such materials may include cell lines, cells disaggregated from tissue, and cells from human or animal blood.

Other preparations from *area postrema* and adjacent tissues include brain slice preparations, brain "chunks," 30 superfused *in situ* preparations, and whole animal preparations where some aspect of *area postrema* response is being measured. These *area postrema* preparations will be used as a readily

available source for development of agonists and antagonists of *area postrema* function. For example, in one embodiment, membranes from cells are obtained by homogenization of cells with an instrument such as Polytron (Brinkman Instruments) followed by centrifugation. Membranes so obtained are combined with labeled known ligand in a buffer system such as that described in Example II, and are incubated and collected as described in that Example. Specific binding of labeled known ligand to the cell membrane or other material used is identified by measuring the decrease in binding obtained in the presence of, for example, 10^{-7} M unlabeled known ligand. Cells in which there is a significant difference between total binding (triplicate tubes) and nonspecific binding (triplicate tubes) at the $P<0.05$ level will be used for further study of *area postrema* component function.

The *area postrema* preparation binding assay described and claimed herein can also be used to further purify *area postrema* components. Membranes are obtained as described in Example II from the *area postrema*. Subcellular membrane fractions obtained by differential or density gradient centrifugation are assayed for specific binding of radiolabeled known ligand in order to identify the membrane fraction containing the highest density of specific *area postrema* component per milligram protein (as assayed by Bradford or Lowry protein assays). The membrane fraction with highest component density is preferably used for further purification.

This membrane fraction is collected and treated in a buffered solution with several membrane solubilizing agents, including triton, digitonin, octyl glucoside, deoxycholate, and cholate, at concentrations of from 0.001% to 1% detergent

at reduced temperature (4°C) for about 1 hour. Protease inhibitors (including phenylmethylsulfonyl fluoride, EDTA, aprotinin) are included in the buffer system to prevent component degradation during or after solubilization. After 5 treatment of membranes with detergents, unsolubilized membranes are sedimented by centrifugation at high speed (100,000 x g for 1 hour) and resulting supernatants containing solubilized components are assayed for binding of radiolabeled metolazone as described above. Solubilized components can be 10 collected by filtration on polyethyleneimine-coated filters (Bruns, R.F., et al. Anal. Biochem. 132:74-81 (1983)). Alternatively, solubilized components are collected by methods such as precipitation with polyethyleneglycol, gel filtration, or equilibrium dialysis. Binding characteristics of 15 solubilized components are assessed and should match the characteristics of membrane-localized components.

After determining desired conditions suitable for solubilizing *area postrema* components and for assaying solubilized components, these solubilized components are 20 purified away from other solubilized membrane proteins by chromatographic procedures, such as affinity chromatography on supports to which known ligand has been coupled, ion exchange chromatography, lectin agarose chromatography, gel filtration, and hydrophobic interaction chromatography. Chromatography 25 column eluates are tested for specific *area postrema* component binding to protein content, in order to identify peaks containing components and the extent of purification. Before inclusion in the final purification protocol, each chromatographic step is tested to determine the extent to 30 which it contributes to component purification, as measured by an increase in specific radiolabelled ligand binding per

milligram protein. Desired chromatography steps are combined sequentially, using large quantities of starting material, in order to obtain partially or completely purified components, as desired.

5 Components which have been partially or completely purified by this method can be used to generate *area postrema*-specific antibodies for use in diagnosis (disease states with altered component density, distribution, or antigenicity) and for use in screening recombinant libraries for *area postrema* 10 component expression. Purified component preparations can also be used to obtain partial sequence information, which is useful in preparing oligonucleotide probes for screening recombinant libraries for *area postrema* component-encoding gene sequences.

15 In another aspect of the invention, changes in cellular response in *area postrema* preparations, or changes in whole-body response attributable to action at *area postrema*, can be used to identify ligands expected to be useful in metabolic conditions. This embodiment differs from those above in that 20 comparison with, or knowledge of, an endogenous ligand is not required. Where *area postrema*-mediated actions are sought in whole animal preparations, assignment of function to *area postrema* can be verified in animals where this tissue has been destroyed by, for example, cautery, radio-frequency lesioning, 25 aspiration, or local application of neurotoxic substances of general or selective toxicity.

Examples

To assist in understanding the present invention the 30 following Examples are included which describe the results of a series of experiments. The experiments relating to this

invention may not, of course, be construed as specifically limiting the invention and such variations of the invention, now known or later developed, which would be within the purview of one skilled in the art are considered to fall 5 within the scope of the invention as described herein and hereinafter claimed.

EXAMPLE I

PREPARATION OF MEMBRANES

10 Membranes are prepared from male Wistar or Sprague-Dawley rats (200-250 grams). Following decapitation brain regions are removed to phosphate-buffered saline (PBS), pH 7.4 at 4EC. Tissues are weighed then placed in 5 ml/g tissue of ice-cold 15 20 mM HEPES buffer, pH 7.4, and homogenized with a Polytron at setting 4 for 10 seconds. An additional 30 ml of cold HEPES is added, and the homogenates centrifuged for 15 minutes at 48,000 x g. After discarding the supernatant fluids, membrane pellets are homogenized in 40 ml of fresh HEPES buffer and centrifuged as before. Membranes are washed again by 20 homogenization in buffer and centrifugation.

 The final membrane pellet is resuspended in a volume of 20 mM HEPES buffer containing 0.2 mM PMSF added immediately before use from a stock 0.2 M solution in ethanol. A volume of buffer is used sufficient to yield a concentration of about 25 0 to about 20 mg original tissue/ml.

EXAMPLE II

BINDING ASSAYS WITH BRAIN MEMBRANES

30 The area postrema is obtained by first cutting the medulla caudally at the obex and rostrally where the floor of the fourth ventricle has just reached its maximum width. Cuts

are then made along the lateral margin of the gracile nucleus and the *area postrema* and underlying tissue removed to a depth of 1-2mm. The membranes are then prepared as in Example I.

Binding assay are carried out by incubating membrane preparations with 0.1-0.3nM labelled ligand, for example, [-¹²⁵I]angiotensin II (1.1-1.8 mCi/ μ g) for 10-15min. After incubation the samples are placed on ice, diluted with 1ml of cold buffer (150mM NaCl, 50mM Tris-HCl, pH 7.2) and filtered through Whatman GF/C glass fibre discs (2.5cm diameter). The filters are washed with 10ml of cold buffer and the radioactivity retained on the filter is determined. Representative procedures and data for this example are based on Sirett, N., et al., Distribution of Angiotensin II Receptors in Rat Brain, Brain Research, 122: 299-312 (1977).

15

¹²⁵ I]Angiotensin II Binding		
<u>Region</u>	<u>(fmoles/mg protein)</u>	<u>Ratio to Cortex</u>
Cortex	.015 +/- 0.05	1
Medulla		
20 <i>Area postrema</i> (A.P.)	1.09 +/- 0.17	7.3
Anterior to A.P.	0.31 +/- 0.08	2.1
Ventral to A.P.	0.76 +/- 0.05	5.1
Anterior Pituitary	3.34 +/- 0.54	22
Adrenals	17.07 +/- 0.91	114

25

EXAMPLE III

WHOLE-ANIMAL VERIFICATION THAT A METABOLICALLY-RELEVANT
RESPONSE RESIDES IN AREA POSTREMA OR STRUCTURES CONNECTED
THERETO

30 The *area postrema* in eight male Sprague Dauley rats, was destroyed by local aspiration following surgical exposure of

art. 31f
andt.

the dorsal medulla oblongata (APX). Seven controls were similarly surgically treated, except the area postrema was left intact (SHAM). Following recovery from surgery, animals were anesthetized with halothane and subjected to a glucose-5 clamp procedure whereby plasma glucose was held constant by a glucose infusion varied in response to frequently determined plasma glucose concentration. After 60 minutes of glucose-clamp, 2mmol L-arginine was infused intravenously over 10 minutes. Plasma glucose, lactate, and insulin were measured 10 for 90 min after L-arginine. There was a large increase in plasma insulin concentration in APX animals that was not observed in SHAM rats. These results demonstrate that pathways controlling insulin secretion, a key hormone involved 15 in fuel homeostasis, include the area postrema.

15 One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The molecular complexes and the methods, procedures, treatments, molecules, specific compounds 20 described herein are presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. ~~Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention are defined by the scope of the claims.~~

25 It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

30 All patents and publications mentioned in the specification are indicative of the levels of those skilled in

the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

5 The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and
10 "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described
15 or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation
20 of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

25 In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

30 The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the

invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

5 Other embodiments are within the following claims.